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(54) Title: DNA SEQUENCING METHOD

(57) Abstract: A method for determining the sequence of a polynucleotide, the method relying on the detection of a conformational change in an enzyme that interacts with and processes along the polynucleotide. The detection of a conformational change may be carried out by measuring changes in a fluorophore bound to the enzyme.

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DNA SEQUENCING METHOD

Field of the Invention

This invention relates to polynucleotide sequence determinations.

5   Background of the invention

The ability to determine the sequence of a polynucleotide is of great scientific importance. For example, the Human Genome Project is an ambitious international effort to map and sequence the three billion
10   bases of DNA encoded in the human genome. When complete, the resulting sequence database will be a tool of unparalleled power for biomedical research. The major obstacle to the successful completion of this project concerns the technology used in the sequencing process.

15   The principle method in general use for large-scale DNA sequencing is the chain termination method. This method was first developed by Sanger and Coulson (Sanger et al., Proc. Natl. Acad. Sci. USA, 1977; 74: 5463-5467), and relies on the use of dideoxy derivatives of the four
20   nucleoside triphosphates which are incorporated into a nascent polynucleotide chain in a polymerase reaction. Upon incorporation, the dideoxy derivatives terminate the polymerase reaction and the products are then separated by gel electrophoresis and analysed to reveal the position at
25   which the particular dideoxy derivative was incorporated into the chain.

Although this method is widely used and produces reliable results, it is recognised that it is slow, labour-intensive and expensive.

30   An alternative sequencing method is proposed in EP-A-0471732, which uses spectroscopic means to detect the incorporation of a nucleotide into a nascent polynucleotide strand complementary to a target. The method relies on an immobilised complex of template and primer, which is
35   exposed to a flow containing only one of the different nucleotides. Spectroscopic techniques are then used to measure a time-dependent signal arising from the polymerase

catalysed growth of the template copy. The spectroscopic
techniques described are surface plasmon resonance (SPR)
spectroscopy, which measures changes in an analyte within
an evanescent wave field, and fluorescence measuring
5   techniques. However, limitations of this method are
recognised; the most serious for the SPR technique being
that, as the size of the copy strand grows, the absolute
size of the signal also grows due to the movement of the
strand out of the evanescent wave field, making it harder
10   to detect increments.   The fluorescence measuring
techniques have the disadvantage of increasing background
interference from the fluorophores incorporated on the
growing nascent polynucleotide chain. As the chain grows,
the background "noise" increases and the time required to
15   detect each nucleotide incorporation needs to be increased.
This severely restricts the use of the method for
sequencing large polynucleotides.
        Single fragment polynucleotide sequencing approaches
are outlined in WO-A-9924797 and WO-A-9833939, both of
20   which employ fluorescent detection of single labelled
nucleotide molecules. These single nucleotides are cleaved
from the template polynucleotide, held in a flow by an
optical trap (Jett, et al., J. Biomol. Struc. Dyn, 1989;
7:301-309), by the action of an exonuclease molecule.
25   These cleaved nucleotides then flow downstream within a
quartz flow cell, are subjected to laser excitation and
then detected by a sensitive detection system. However,
limitations of this method are recognised; the most serious
for the exonuclease technique being the fact that the
30   labelled nucleotides severely affect the processivity of
the exonuclease enzyme. Other limitations of this method
include 'sticking' of the nucleotide(s) to the biotin bead
used to immobilise the polynucleotide fragment, thus
resulting in the nucleotide flow becoming out of phase;
35   inefficiency and length limitation of the initial enzymatic
labelling process; and the excitation 'cross-over' between

the four different dye molecules resulting in a greatly increased error rate.

There is therefore a need for an improved method, preferably at the single fragment level, for determining the sequence of polynucleotides, which significantly increases the rate and fragment size of the polynucleotide sequenced and which is preferably carried out by an automated process, reducing the complexity and cost associated with existing methods.

Summary of the Invention

The present invention is based on the realisation that the sequence of a target polynucleotide can be determined by measuring conformational changes in an enzyme that binds to and processes along the target polynucleotide. The extent of the conformational change that takes place is different depending on which individual nucleotide on the target is in contact with the enzyme.

According to one aspect of the present invention, a method for determining the sequence of a polynucleotide comprises the steps of:

(i) reacting a target polynucleotide with an enzyme that is capable of interacting with and precessing along the polynucleotide, under conditions sufficient to induce the enzyme activity; and

(ii) detecting conformational changes in the enzyme as the enzyme processes along the polynucleotide.

In a preferred embodiment, the enzyme is a polymerase enzyme which interacts with the target in the process of extending a complementary strand. The enzyme is typically immobilised on a solid support to localise the reaction within a defined area.

According to a second embodiment of the invention, the enzyme comprises a first bound detectable label, the characteristics of which alter as the enzyme undergoes a conformational change. The enzyme may also comprise a second bound detectable label capable of interacting with the first label, wherein the degree of interaction is

dependent on a conformational change in the enzyme. Typically, the first label is an energy acceptor and the second label is an energy donor, and detecting the conformational change is carried out by measuring energy transfer between the two labels.

According to a further embodiment of the invention, fluorescence resonance energy transfer (FRET) is used to detect a conformational change in an enzyme that interacts with and processes along a target polymerase, thereby determining the sequence of the polynucleotide. Fluorescence resonance energy transfer may be carried out between FRET donor and acceptor labels, each bound to the enzyme. Alternatively, one of the labels may be bound to the enzyme and the other label bound to the polynucleotide.

According to a further embodiment, there is the use of a detectably-labelled enzyme, capable of interacting with and precessing along a target polynucleotide, to determine the sequence of the polynucleotide, wherein the label alters its detectable characteristics as the enzyme processes along the polynucleotide.

According to a further aspect, a solid support comprises at least one immobilised enzyme capable of interacting with and precessing along a target polynucleotide, the enzyme being labelled with one or more detectable labels.

According to a further aspect, a system for determining the sequence of a polynucleotide comprises a solid support as defined above, and an apparatus for detecting the label.

The present invention offers several advantages over conventional sequencing technology. Once a polymerase enzyme begins its round of polynucleotide elongation, it tends to polymerase several thousand nucleotides before falling off from the strand. Additionally, certain specific polymerase systems are able to anchor or tether themselves to the template polynucleotide via a 'sliding clamp' (e.g. Polymerase III) which encircles the template

molecule or via a molecular hook (e.g. T7:thireodoxin complex) which partially encircles the template.

The invention may also enable tens of kilobases (kb) or more to be sequenced in one go, at a rate of hundreds of base pairs per second. This is a result of sequencing on a single fragment of DNA. An advantage of sequencing a single fragment of DNA is that sequencing rates are determined by the enzyme system utilised and not upon indirect, summated reactions, and are therefore correspondingly higher. Just as important as the high rate is the ability to sequence large fragments of DNA. This will significantly reduce the amount of subcloning and the number of overlapping sequences required to assemble megabase segments of sequencing information. An additional advantage of the single fragment approach is the elimination of problems associated with the disposal of hazardous wastes, such as acrylamide, which plague current sequencing efforts.

Description of the Drawings

Figure 1 is a schematic illustration of a confocal microscope setup for use in the invention;

Figure 2 illustrates a trace taken after fluorescence resonance energy transfer, with each of the peaks representing the detection of a specific nucleotide.

Description of the Invention

The present method for sequencing a polynucleotide involves the analysis of conformational changes between an enzyme and a target polynucleotide.

The term "polynucleotide" as used herein is to be interpreted broadly, and includes DNA and RNA, including modified DNA and RNA, as well as other hybridising nucleic acid-like molecules, e.g. peptide nucleic acid (PNA).

The enzyme may be a polymerase enzyme, and a conformational change is brought about when the polymerase incorporates a nucleotide into a nascent strand complementary to the target polynucleotide. It has been found that the conformational change will be different for

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each of the different nucleotides, A, T, G or C and therefore measuring the change will identify which nucleotide is incorporated.

Alternatively, the enzyme may be any that is involved in an interaction with a polynucleotide, e.g. a helicase enzyme, primase and holoenzyme. As the enzyme processes along the polynucleotide, its conformation will change depending on which nucleotide on the target it is brought into contact with.

One way of detecting a conformational change in the enzyme is to measure resonance energy transfer between a suitable energy donor label and a suitable energy acceptor label. In one example the donor and acceptor are each bound to the enzyme and the conformational change in the enzyme brought about by its interaction with the target polynucleotide alters the relative positioning of the labels. The differences in positioning are reflected in the resulting energy transfer and are characteristic of the particular nucleotide in contact with the enzyme. Alternatively, one label may be positioned on the enzyme and the other on a nucleotide of the target or on a nucleotide incorporated onto a strand complementary to the target.

The use of fluorescence resonance energy transfer (FRET) is a preferred embodiment of the invention. This technique is capable of measuring distances on the 2- to 8nm scale and relies on the distance-dependent energy transfer between a donor fluorophore and an acceptor fluorophore. The technique not only has superior static co-localization capabilities but can also provide information on dynamic changes in the distance or orientation between the two fluorophores for intramolecular and intermolecular FRET. Since the first measurement of energy transfer between a single donor and a single acceptor (single pair FRET) (Ha, et al., Proc. Natl. Acad. Sci. USA, 1996; 96:893), it has been used to study ligand-receptor co-localisation (Schutz, et al., Biophys. J.,

1998; 74:2223), to probe equilibrium protein structural
fluctuations and enzyme-substrate interactions during
catalysis (Ha, et al., 1999 Supra), and to identify
conformational states and sub-populations of individual
5   diffusing molecules in solutions. All of these variables
are envisioned as applicable within the context of the
invention.

The present invention may also be carried out using
measurement techniques that require only a single label.
10   Any system that is capable of measuring changes in the
local environment of the enzyme at the single molecule
level, is an accepted embodiment of the invention. Various
properties of single fluorescent probes attached to a
polynucleotide processive enzyme and/or its substrate(s)
15   can be exploited in the context of the invention to provide
data on variables within or in close proximity to the
enzyme system/molecular environment that are specific to a
nucleotide incorporation event. Such variables include,
but are not limited to, molecular interactions, enzymatic
20   activity, reaction kinetics, conformational dynamics,
molecular freedom of motion, and alterations in activity
and in chemical and electrostatic environment.

For example, the absorption and emission transition
dipoles of single fluorophores can be determined by using
25   polarized excitation light or by analysing the emission
polarisation, or both. The temporal variation in dipole
orientation of a rigidly attached or rotationally diffusing
tethered label can report on the angular motion of a
macromolecule system or one of its subunits (Warshaw, et
30   al., Proc. Natl. Acad. Sci. USA, 1998; 95:8034) and
therefore may be applied in the present invention.

The labels that may be used in the present invention
will be apparent to those skilled in the art. Preferably,
the label is a fluorescence label, such as those disclosed
35   in Xue, et al., Nature, 1995; 373:681. Alternatively,
fluorescing enzymes such as green fluorescent protein (Lu,
et al., Science, 1998; 282:1877) can be employed.

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The preferred embodiment of the invention, however, involves the use of small fluorescence molecules that are covalently and site-specifically attached to the polynucleotide processive enzyme, e.g. tetramethylrhodamine

5 (TMR).

If fluorescent labels are used in the invention, their detection may be affected by photobleaching caused by repeated exposure to excitation wavelengths. One possible way to avoid this problem is to carry out many sequential

10 reactions, but detecting fluorescence signals on only a few at a time. Using this iterative process, the correct sequence of signals can be determined and the polynucleotide sequence determined. For example, by immobilising a plurality of enzymes on a solid support and

15 contacting them with the target polynucleotide, the sequencing reactions should start at approximately the same time. Excitation and detection of fluorescence can be localised to a proportion of the total reactions, for a time until photobleaching becomes evident. At this time,

20 excitation and detection can be transferred to a different proportion of the reactions to continue the sequencing. As all the reactions are relatively in phase, the correct sequence should be obtained with minimal sequence re-assembly.

25 The labels may be attached to the enzymes by covalent or other linkages. A number of strategies may be used to attach the labels to the enzyme. Strategies include the use of site-directed mutagenesis and unnatural amino acid mutagenesis (Anthony-Cahil, *et al.*, Trends Biochem. Sci.,

30 1989; 14:400) to introduce cysteine and ketone handles for specific and orthogonal dye labelling proteins (Cornish, *et al.*, Proc. Natl. Acad. Sci. USA, 1994; 91:2910).

Another foreseen embodiment used to tag the polynucleotide processive enzyme is the fusion of green

35 fluorescent protein (GFP) to the processive enzyme (e.g. polymerase) via molecular cloning techniques known in the art (Pierce, D.W. *et al.*, Nature, 1997; 388:338). This

technique has been demonstrated to be applicable to the measurement of conformational changes (Miyawaki, et al., Nature, 1997; 388:882) and local pH changes (Llopis, et al., Proc. Natl. Acad. Sci. USA, 1998; 95:6803).

5   Supports suitable for use in immobilising the enzymes, will be apparent to the skilled person. Silicon, glass and ceramics materials may be used. The support will usually be a planar surface. Enzyme immobilisation may be carried out by covalent or other means. For example, covalent

10   linker molecules may be used to attach to a suitably prepared enzyme. Attachment methods are known to the skilled person.

There may be one or more enzymes immobilised to the solid support. In a preferred embodiment, there are a

15   plurality of enzymes attached. This allows monitoring of many separate reactions, and may be useful to overcome photobleaching problems as outlined above.

A variety of techniques may be used to measure a conformational change in the enzyme. Resonance energy

20   transfer may be measured by the techniques of surface plasmon resonance (SPR) or fluorescent surface plasmon resonance.

However, other techniques which measure changes in radiation via interaction with a 'label' or energy

25   transducer may be considered, for example spectroscopy by total internal reflectance fluorescence (TIRF), attenuated total reflection (ATR), frustrated total reflection (FTR), Brewster angle reflectometry, scattered total internal reflection (STIR), fluorescence lifetime imaging microscopy

30   and spectroscopy (FLIMS), fluorescence polarisation anisotrophy (FPA), fluorescence spectroscopy, or evanescent wave ellipsometry.

The invention will now be illustrated further by the following Example, with reference to the accompanying

35   drawings.

10

Example

This Example used a confocal fluorescence setup, as shown in Fig. 1.

With reference to Fig. 1, the setup consists of a scan table (1) able to scan at high resolution in X, Y and Z dimensions, a class coverslip (2) which is part of a microfluidic flow cell system with an inlet (8) for introducing the primer-template polynucleotide complex (4) and nucleotides over the immobilised (9) polymerase molecule (3) within a buffer, and an outlet (7) for waste. Incident light from a laser light source (6) for donor excitation is delivered via an oil-immersion objective (5).

Protein Conjugation

In this experiment, Tetramethylrhodamine (TMR, donor) and Cy5 (acceptor) where used as the FRET pair. This was due to their well separated emission wavelengths (>100nm) and large Föster radius.

T7 DNA Polymerase from New England Biolabs (supplied at 10 000 U/ml) was used. 50μl of T7 was buffer-exchanged in a Vivaspin 500 (Vivaspin) against 4 x 500μl of 200mM Sodium Acetate buffer at pH 4 in order to remove the DTT from the storage buffer that the T7 DNA Polymerase is supplied in. Then, 50μl of the buffer-exchanged T7 DNA polymerase was added to 100μl of Sodium Acetate buffer at pH 4 and 50 μl saturated 2-2-DiPyridyl-DiSulphide in aqueous solution. This reaction was then left for 110 minutes and the absorption at 343nm noted. Finally, the sample was then buffer-exchanged into 200mM Tris at pH 8 as before (4 times 500μl).

Dye attachment was verified by denaturing polyacrylamide gel electrophoresis. Cy5 succinimidyl ester (Molecular Probes) was conjugated to the TMR-T7 DNA Polymerase under the same labelling conditions and purified and characterized as described above.

Polymerase Immobilization

Glass coverslips were derivatized with N-[(3-trimethoxysilyl)propyl] ethylenediamine triacetic acid.

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The coverslip was then glued into a flow-cell arrangement that allowed buffer to be flowed continuously over the derivatized glass surface. The labelled polymerase was then added to the buffer and allowed to flow over the

5   coverslip so that protein was immobilised on the glass surface.

Proteins were then immobilised on the glass-water interface with low density so that only one molecule was under the laser excitation volume at any one time. Laser

10   light (514 nm Ar ion laser, 15 μW, circularly polarized) was focused onto a 0.4 μm spot using an oil immersion objective in an epi-illumination setup of a scanning-stage confocal microscope. The fluorescence emission was collected by the same objective and divided into two by a

15   dichroic beam splitter (long pass at 630 nm) and detected by two Avalanche Photo Diode (APD) counting units, simultaneously.

A 585 nm band pass filter was placed in front of the donor detector; a 650 nm long pass filter was placed in

20   front of the acceptor detector. Since the spectral ranges during fluorescence detection are sufficiently removed from the cutoff wavelength of the dichroic beam splitter, the polarization dependence of the detection efficiency of both donor and acceptor signal is negligible. It has been shown

25   that the polarization mixing due to the high near field aperture (NA) objective can be overlooked (Ha et al, Supra).

In order to acquire donor and acceptor emission times, a search condition on the acceptor signal was employed as

30   outlined in (Ha, et al., Appl. Phys. Lett., 1997; 70:782). This procedure aids in the screening of doubly labelled proteins: with no direct excitation of the acceptor, only proteins experiencing FRET could show acceptor signal. Once a protein was screened, located and positioned under

35   the laser spot, donor and acceptor time traces (5 ms integration time) were acquired. The acquisition time lasted until all the fluorescent labels on the target

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protein where photobleached.

Reaction Initiation

　　　Two oligonucleotides were synthesised using standard
phosphoramidite chemistry. The oligonucleotide defined as
5   SEQ ID NO.1 was used as the target polynucleotide, and the
oligonucleotide defined as SEQ ID No.2 was used as the
primer. The two oligonucleotides were reacted under
hybridizing conditions to form the target-primer complex.

10   CAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGAGAGAAGGGAGAG
                                     SEQ ID NO.1

     CTCTCCCTTCTCTCGTC   SEQ ID NO.2

15   　　　The reaction was then initiated by injecting the
primed DNA into the flow cell with all four nucleotides
(dGTP, dCTP, dATP and dTTP) present at a concentration of
0.4mM. The flow cell was maintained at 25 degrees Celsius
by a modified peltier device.

20   　　　An oxygen-scavenging system was also employed [50
μg/ml glucose oxidase, 10 μg/ml catalase, 18% (wt/wt)
glucose, 1% (wt/vol) β-mercaptoethanol] to prolong
fluorescent lifetimes (Funatsu, et al., Nature, 1995;
374:555-559).

25   FRET data anaylsis

　　　Initial studies have determined the origins of
blinking, photobleaching and triplet state spikes (Ha, et
al., Chem. Phy., 1999; 247:107-118), all of which can
interfere with the underlying changes in FRET efficiency,
30   due to distance changes between fluorophores as a result of
conformational changes. After subtracting the background
signal from donor and acceptor time traces as disclosed in
Ha, et al., 1999 (Supra), a median filter, with five points
average, was applied to remove triplet spikes. Next, data
35   points that showed simultaneous dark counts on both
detectors due to donor blinking events were disregarded
from the time traces.

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The amount of donor signal recovery upon acceptor photobleaching is related to the quantum yields of the molecules and their overall detection efficiencies.

Energy transfer efficiency time trace was then
5   obtained.   The FRET efficiency time trace during polymerization of target strand SEQ ID NO.1 shown in figure 2.   Reading Fig. 2, the sequence corresponds to the complement of that of SEQ ID No. 1 (reading right to left, minus that part which hybridises to the primer sequence).

10

CLAIMS

1.   A method for determining the sequence of a polynucleotide, comprising the steps of:

    i.   reacting a target polynucleotide with an enzyme that is capable of interacting with and precessing along the polynucleotide, under conditions sufficient to induce enzyme activity; and

    ii.   detecting conformational changes in the enzyme as the enzyme processes along the polynucleotide.

2.   A method according to claim 1, wherein the enzyme is a polymerase enzyme.

3.   A method according to claim 1, wherein the enzyme is a helicase enzyme or a primase enzyme.

4.   A method according to any preceding claim, wherein the enzyme is immobilised on a solid support.

5.   A method according to claim 4, comprising a plurality of enzymes immobilised on the solid support.

6.   A method according to any preceding claim, wherein the enzyme comprises a first bound detectable label, the characteristics of which alter as the enzyme undergoes a conformational change.

7.   A method according to claim 6, wherein the enzyme comprises a second bound detectable label capable of interacting with the first label, wherein the degree of interaction is dependent on a conformational change in the enzyme.

8.   A method according to claim 6, wherein a second detectable label is bound to a nucleotide brought into contact with the enzyme.

9.   A method according to claim 7 or claim 8, wherein the first label is an energy acceptor and the second label is an energy donor, or vice versa, and wherein step (ii) is carried out by measuring energy transfer between the two labels.

10.   A method according to any preceding claim, wherein step (ii) is carried out using confocal microscopy.

11.   A method according to claim 10, wherein step (ii) is

15

carried out by fluorescence imaging.

12. A method according to claim 6, wherein step (ii) is carried out by measuring a polarisation effect consequent on the altered characteristics of the first label.

5   13. A method according to claim 12, wherein step (ii) is carried out by fluorescence polarisation anisotrophy.

14. Use of fluorescence resonance energy transfer to detect a conformational change in an enzyme that interacts with and processes along a target polymerase, to thereby

10   determine the sequence of the polynucleotide.

15. Use according to claim 14, wherein the enzyme is a polymerase enzyme.

16. Use according to claim 14 or claim 15, wherein the enzyme is immobilised on a solid support.

15   17. Use of a detectably-labelled enzyme, capable of interacting with and precessing along a target polynucleotide, to determine the sequence of the polynucleotide, wherein the label alters its detectable characteristics as the enzyme processes along the

20   polynucleotide.

18. A solid support comprising at least one immobilised enzyme capable of interacting with and precessing along a target polynucleotide, the enzyme being labelled with one or more detectable labels.

25   19. A solid support according to claim 18, wherein the enzyme is a polymerase.

20. A solid support according to claim 18 or claim 19, wherein the label is a fluorophore.

21. A system for determining a sequence of a

30   polynucleotide, comprising a solid support according to any of claims 18 to 21, and an apparatus for detecting the label.
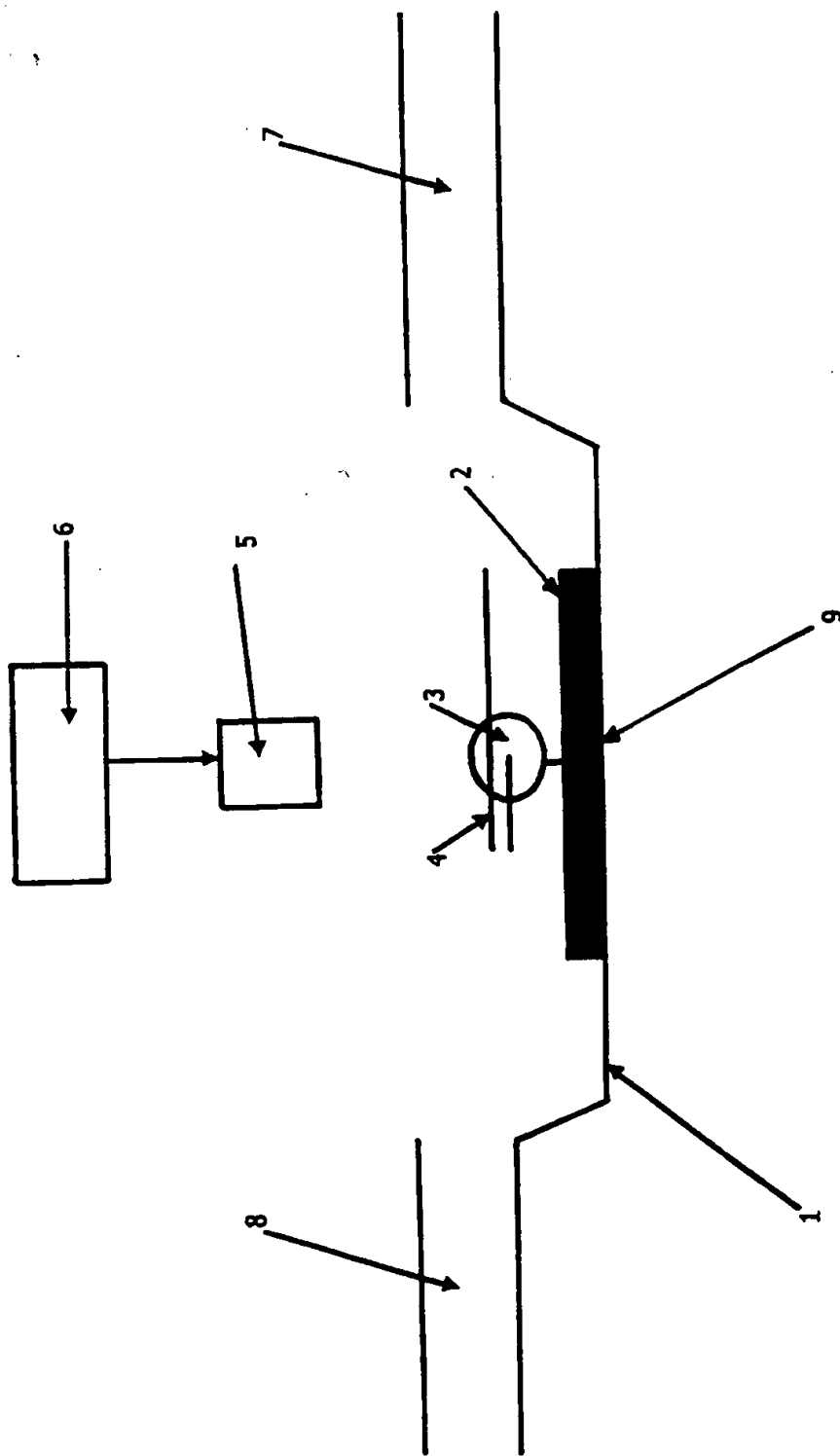
1 / 2

Figure 1

Figure 2

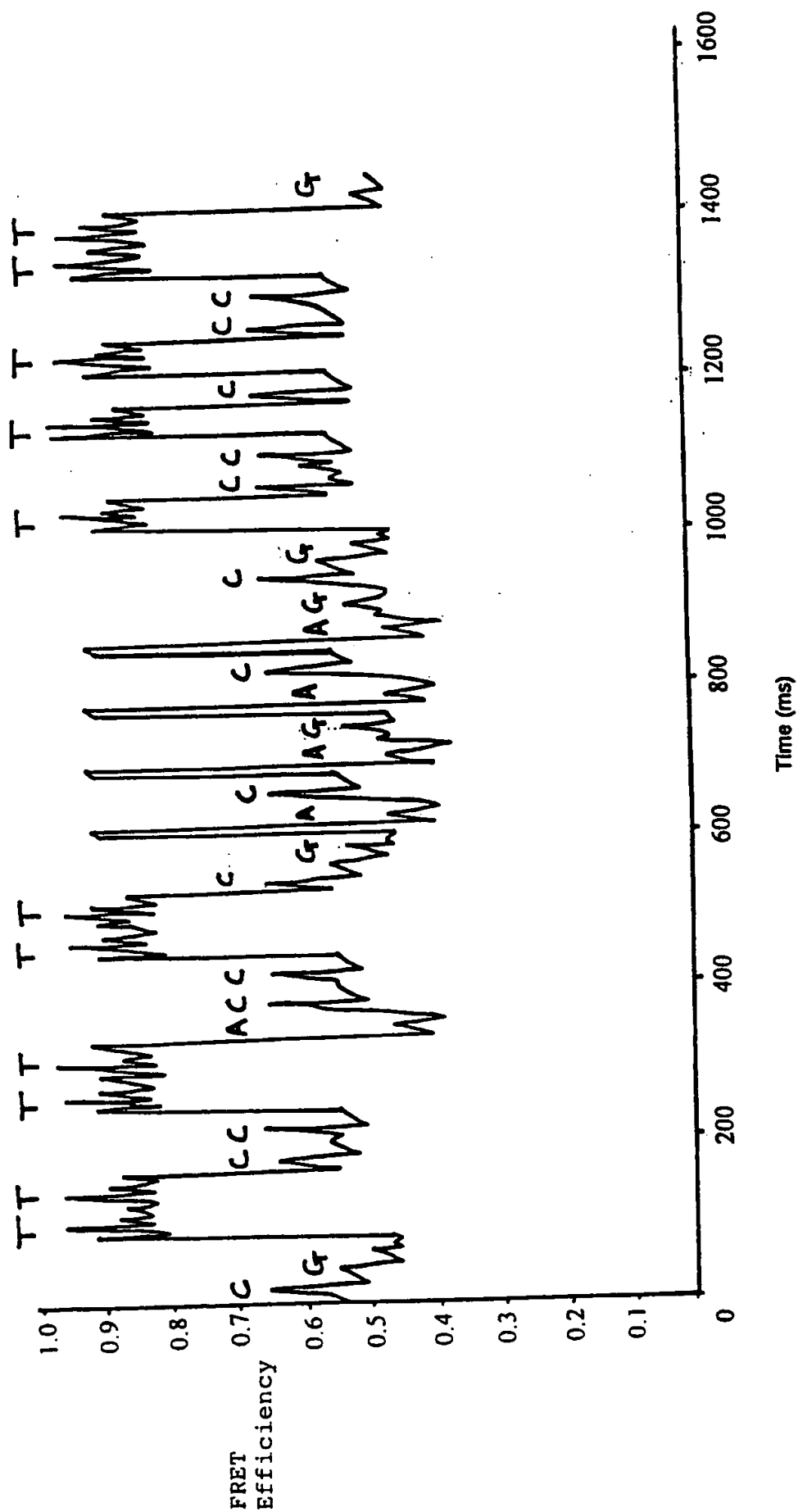SEQUENCE LISTING

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